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Oxygenation of the Endocannabinoid, 2-Arachidonylglycerol, to Glyceryl Prostaglandins by Cyclooxygenase-2*

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Cyclooxygenases (COX) play an important role in lipid signaling by oxygenating arachidonic acid to endoperoxide precursors of prostaglandins and thromboxane. Two cyclooxygenases exist which differ in tissue distribution and regulation but otherwise carry out identical chemical functions. The neutral arachidonate derivative, 2-arachidonylglycerol (2-AG), is one of two described endocannabinoids and appears to be a ligand for both the central (CB1) and peripheral (CB2) cannabinoid receptors. Here we report that 2-AG is a substrate for COX-2 and that it is metabolized as effectively as arachidonic acid. COX-2-mediated 2-AG oxygenation provides the novel lipid, prostaglandin H₂ glycerol ester (PGH₂-G), *in vitro* and in cultured macrophages. PGH₂-G produced by macrophages is a substrate for cellular PGD synthase, affording PGD₂-G. Pharmacological studies reveal that macrophage production of PGD₂-G from endogenous sources of 2-AG is calcium-dependent and mediated by diacylglycerol lipase and COX-2. These results identify a distinct function for COX-2 in endocannabinoid metabolism and in the generation of a new family of prostaglandins derived from diacylglycerol and 2-AG.

Cyclooxygenase (COX¹; prostaglandin endoperoxide synthase, EC 1.14.99.1) catalyzes the *bis*-dioxygenation of arachidonic acid, generating prostaglandin (PG) H₂, the precursor to a diverse family of lipid mediators including PGs, thromboxane, and prostacyclin (1). The discovery of a second COX isoform, COX-2, has provided important insights into the molecular basis of inflammation, hyperalgesia, and cancer and has established a novel pharmacological target for their treatment (2–4). The major functional differences between COX-1 and COX-2 are believed to be related to their differential regulation and tissue distribution (5). COX-1 is a constitutive enzyme,

whereas COX-2 is inducible and highly regulated by a range of agonists (6, 7). COX-1 activity accounts for PG and thromboxane production in gastric mucosa, kidney, and platelets (3). COX-2 activity is primarily responsible for PG biosynthesis in the central nervous system and inflammatory cells (8–10). These observations suggest that COX-1 and COX-2 serve different physiological and pathophysiological functions.

The possibility that COX-2 has distinct biochemical functions has not been explored extensively. There are conserved structural differences between the active sites of COX-1 and COX-2 which have been exploited in the development of selective COX-2 inhibitors. It is possible that these or other structural differences may have evolved to support separate biochemical functions for the two COX isoforms. The first indication of a separate biochemical function for COX-2 was provided by the observation that it selectively oxygenates the neutral ethanolamide derivative of arachidonic acid, anandamide (11). Anandamide and 2-arachidonylglycerol (2-AG) are endogenous ligands for the cannabinoid receptors that bind Δ⁹-tetrahydrocannabinol and mediate its pharmacological effects (12). The cannabimimetic properties of 2-AG were first reported in 1995, and subsequent investigations demonstrated that this monoglyceride is present *in vivo* at concentrations several orders of magnitude higher than anandamide (13–15). Extensive structure-activity studies suggest that 2-AG is the intrinsic natural ligand for the peripheral cannabinoid (CB2) receptor, widely expressed in immune tissues, and a physiological ligand for the central cannabinoid (CB1) receptor (16, 17). The biochemical pathways for the generation of 2-AG are distinct from those responsible for anandamide biosynthesis and involve diacylglycerol production and hydrolysis (18) (Structures 1–3).

Several observations prompted us to investigate the ability of COX-2 to utilize 2-AG as a substrate. The expression of COX-2 in the central nervous and immune systems parallels the distribution of the CB1 and CB2 receptors, respectively (7, 12). 2-AG is present at high levels in regions of the brain and in immune cells where COX-2 is expressed, and 2-AG appears to be unique in its ability to activate efficiently both known cannabinoid receptors (14, 18, 19). Finally, neutralization of the carboxylic acid of the nonsteroidal antiinflammatory agent, indomethacin, by esterification results in potent, highly selective COX-2 inhibitors, suggesting that arachidonyl esters may serve as COX-2 selective substrates (20).

We report here that COX-2 oxygenates 2-AG as effectively as arachidonic acid. COX-2-mediated 2-AG oxygenation was demonstrated with purified enzyme and with intact macrophages. The products of 2-AG oxygenation are PG and hydroxyeicosatetraenoic acid (HETE) esters that represent the first entries into a novel class of glyceryl eicosanoids. Site-directed mutagenesis indicates that conserved structural differences between COX-1 and COX-2 contribute significantly to the ability

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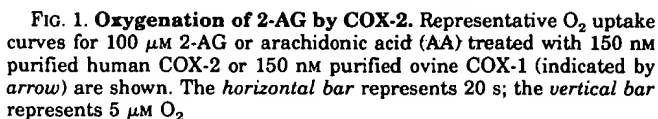
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¹ The abbreviations used are: COX, cyclooxygenase; PG, prostaglandin; AG, arachidonylglycerol; CB1 and CB2, central and peripheral cannabinoid, respectively; HETE, hydroxyeicosatetraenoic acid; DAG, diacylglycerol; MS, mass spectrometry; G, glyceryl ester; HPLC, high performance liquid chromatography; LC, liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; IFN, interferon; LPS, lipopolysaccharide.



EXPERIMENTAL PROCEDURES

Mass Spectrometry—Liquid chromatography (LC)/MS was conducted with a Waters 2690 Separations Module with a Zorbax RX-C18 narrow bore column (15 cm \times 2.1 mm, 5 μ m) interfaced to a Finnigan TSQ-7000 triple quadrupole mass spectrometer. Sodiated analytes were eluted with increasing concentrations of MeCN in 0.001% aqueous NaOAc. Evaluation of glyceryl prostaglandin biosynthesis by RAW264.7 cells was conducted with selected ion monitoring, and quantitation was accomplished using pentadeuterated glyceryl prostaglandin standards.



Endogenous 2-AG Production and Metabolism by RAW264.7 Cells—Low passage number murine RAW264.7 cells were stimulated with IFN- γ and LPS as described above. Activated RAW264.7 cell medium was aspirated and replaced with fresh serum-free DMEM. Cells in a single well of a six-well plate were treated with dimethyl sulfoxide vehicle, indomethacin (3 μ M), U-73122 (5 μ M), or RHC-80267 (100 μ M) for 20 min at 37 $^{\circ}$ C followed by the addition of dimethyl sulfoxide vehicle or ionomycin (5 μ M). After 20 min, medium was removed and treated with 8.0 ng of pentadeuterated glyceryl prostaglandin standard. Medium was extracted twice with an equal volume of CHCl_3 :MeOH (2:1). The solvent was evaporated, and the residue was redissolved in H_2O :MeCN (1:1), filtered (0.22- μ m nylon), and subjected to LC/MS analysis.

RESULTS

Oxygenation of 2-AG by Purified Human and Mouse COX-2—Incubation of 2-AG with purified recombinant human COX-2 triggered O₂ uptake comparable in rate and extent to that observed with arachidonic acid (Fig. 1). In contrast, relatively little O₂ uptake was observed after the addition of sheep COX-1 to 2-AG. Steady-state kinetic analysis revealed that both human COX-2 and mouse COX-2 oxygenated 2-AG with apparent k_{cat}/K_m values similar to those determined for arachidonic acid (Table I). The k_{cat}/K_m of human COX-2 for 2-AG is 60-fold higher than the value of 0.065 s⁻¹ μM⁻¹ reported for anandamide (27). Among a series of arachidonyl esters, 2-AG was clearly the preferred substrate (Fig. 2). The initial rate of 2-AG oxygenation displayed in Fig. 2 is approximately half the

initial rate of arachidonic acid oxygenation in agreement with the k_{cat} values determined for both substrates with murine COX-2 (Table I). Of particular interest, COX-2 oxygenated the more stable arachidonylglycerol regioisomer, 1-AG, at a markedly reduced rate. In addition, DAG, the biosynthetic precursor for 2-AG, and the related esters, 1,2-diarachidonylglycerol and 1,3-diarachidonylglycerol, were very poor substrates for COX-2 (Fig. 2).

Characterization of Oxygenated 2-AG Metabolites—The products of 2-AG oxygenation were identified by MS. Organic extracts of reaction mixtures containing human COX-2 and 2-AG were infused directly into a triple quadrupole mass spectrometer and analyzed following electrospray ionization. Two primary product masses were observed at m/z 449 and 417, which correspond to the sodiated molecular ions of the glyceryl esters of PGE_2 , PGE_2 , or PGD_2 , and 11- or 15-HETE, respectively (Fig. 3a and Scheme 1). The presence of sodium in the molecular ions was confirmed by collision-induced dissociation. Treatment of 2-AG/COX-2 incubation mixtures with endoperoxide reducing agents (triphenylphosphine, SnCl_2 , or $\text{Na}_2\text{S}_2\text{O}_4$) resulted in the disappearance of the ion at m/z 449 and the appearance of a product with a molecular ion at m/z 451 (Fig. 3b). This is consistent with reduction of PGH_2 -G to $\text{PGF}_{2\alpha}$ -G. Another peak was observed at m/z 377 in reaction mixtures quenched with $\text{Na}_2\text{S}_2\text{O}_4$ (Fig. 3b). This mass corresponds to the sodiated form of the glyceryl ester of 12-hydroxyheptadeca-5,8,10-trienoic acid (HHT-G, Scheme 1).

LC/MS revealed the presence of four primary products in 2-AG/COX-2 reaction mixtures. Two closely eluting polar products each displayed m/z 449 consistent with PGE_2 -G and PGD_2 -G (Fig. 4a). Pentadeuterated standards of glyceryl esters of PGE_2 and PGD_2 were synthesized and coeluted with these two polar products under multiple chromatographic conditions. In addition, two closely eluting nonpolar 2-AG metabolites were detected with an m/z of 417 consistent with the glyceryl

esters of HETEs (Fig. 4b). Finally, a minor product of intermediate polarity and m/z 377 was observed, which is consistent with HHT-G (data not shown).

Estimates of relative amounts of the individual products were made by reversed-phase HPLC with UV detection. The two polar products with m/z 449 exhibited no significant absorption above 215 nm, whereas both the intermediate polarity, minor product and the two nonpolar products exhibited absorption maxima near 235 nm. The 235 nm absorbance maximum indicates the presence of a conjugated diene functionality. Base treatment of the oxygenated 2-AG metabolites afforded free acids that coeluted with standards of 15- and 11-HETE and the PGE_2 dehydration/isomerization product, PGB_2 , which displayed an absorption maximum at 278 nm (Reaction 1). Relative quantitation of PGE_2 -G and HETE-Gs was accomplished by HPLC/UV analysis of base-treated COX-2/2-AG incubation mixtures using 5-HETE as an internal standard. PGE_2 -G was the major product of oxygenation (PGE_2 -G/HETE-G = 4.0 ± 0.1 , $n = 5$) and 11-HETE-G was more abundant than 15-HETE-G (11-HETE-G/15-HETE-G = 3.4 ± 0.1 , $n = 5$).

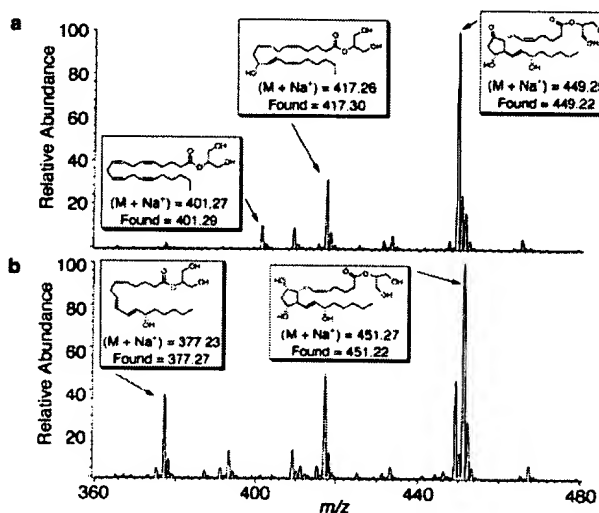


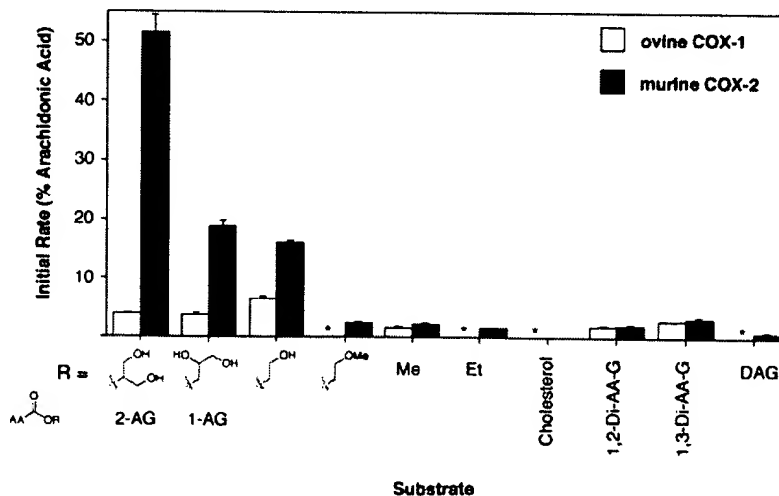
FIG. 3. Mass spectrometry of oxygenated 2-AG products. Representative direct liquid infusion, positive ion, electrospray ionization mass spectra of 2-AG metabolites produced by treating 15 μg 2-AG with (panel a) 15 μg of purified human COX-2 for 2 min at 37 °C or (panel b) 30 μg of purified human COX-2 for 2 min at 37 °C followed by reduction with 15% $\text{Na}_2\text{S}_2\text{O}_4$ for 20 min at room temperature are shown. Chemical structures indicate the assignment for the most abundant product with the appropriate mass-to-charge ratio.

TABLE I
Steady-state kinetic parameters for COX-2 mediated 2-AG and arachidonic acid oxygenation

	k_{cat} s^{-1}	K_m μM	k_{cat}/K_m $\text{s}^{-1} \mu\text{M}^{-1}$
Human COX-2			
AA ^a	14.7 ± 0.5	6.1 ± 0.6	2.4
2-AG	17.4 ± 1.1	4.4 ± 0.9	4.0
Murine COX-2			
AA	20.5 ± 1.6	8.2 ± 1.6	2.5
2-AG	11.1 ± 0.7	4.7 ± 0.8	2.3

^a AA, arachidonic acid.

FIG. 2. Specificity of oxygenation of arachidonyl esters. Initial O_2 uptake rates of arachidonyl esters (200 μM) by murine COX-2 (200 nM) and ovine COX-1 (150 nM) are shown and are normalized to the initial rate of O_2 uptake for arachidonic acid (100 μM) (mean \pm S.E., $n \geq 3$). Asterisks indicate a substrate that was not evaluated with COX-1. Di-AA-G, diarachidonylglycerol.



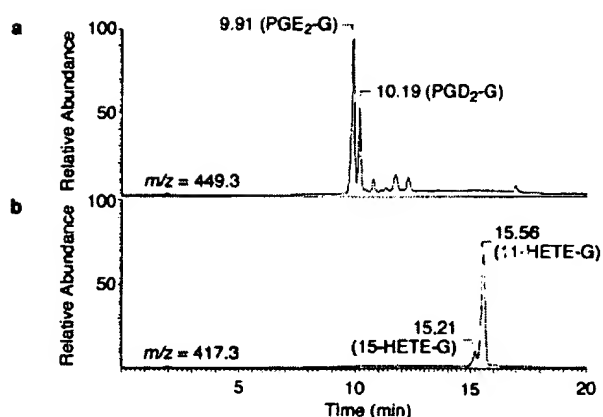
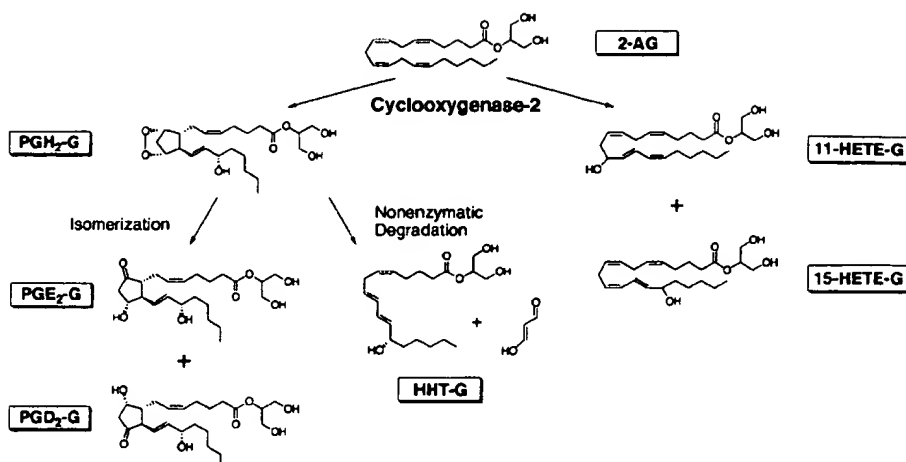
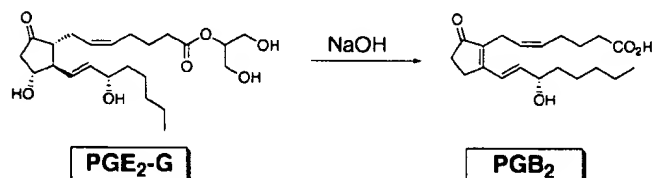
SCHEME 1. *In vitro* transformations of 2-AG.

FIG. 4. Representative reversed phase liquid chromatography-mass spectrometry selected-ion chromatograms of oxygenated 2-AG metabolites with $m/z = 449.3$ (panel a) and $m/z = 417.3$ (panel b). Products were eluted with a 15-min gradient of 20–100% acetonitrile in H₂O (0.001% sodium acetate).

HETE-G regiochemistry was confirmed by mass spectrometry of saponified, HPLC-purified HETE products which demonstrated collision-induced dissociation patterns consistent with previous reports for 11- and 15-HETE (28). This product profile for 2-AG oxygenation is similar to that of arachidonic acid oxygenation by COX-2 (29).



REACTION 1

Enzyme Structural Requirements for 2-AG Oxygenation—

The structural requirements for 2-AG oxygenation by COX-2 were probed by site-directed mutagenesis using murine proteins expressed in insect cells from baculovirus vectors and purified to homogeneity. An active site tyrosyl radical formed from Tyr-385 initiates arachidonic acid oxygenation by 13-pro-S hydrogen abstraction, and consequently, the Y385F mutant enzyme fails to oxidize arachidonic acid (30). As expected, no oxygen uptake was detectable when 2-AG was incubated with Y385F mCOX-2. Arachidonic acid binds in the COX active site in an L-shaped conformation with the ω -end inserted in a

hydrophobic channel near Gly-533 (Fig. 5) (23, 31). Mutations that introduce steric bulk at position 533 prevent arachidonate binding and oxygenation (23). Consistent with previous results with arachidonic acid, no oxygen uptake was detected when 2-AG was incubated with the G533V enzyme. Arg-120 has been shown to ion-pair with the carboxylate moieties of both arachidonate and acidic non-steroidal antiinflammatory drugs (32, 33). Substitution of Arg-120 with glutamine in mCOX-2 reduced the maximal rate of 2-AG oxygenation to $11 \pm 1\%$ ($n = 3$) of the wild-type rate. This result was somewhat surprising because 2-AG does not contain a free carboxylate. Nevertheless, taken together, the results suggest that 2-AG binds within the COX-2 active site in a conformation similar to arachidonic acid with the ω -end positioned in the hydrophobic top channel, the polar head group near Arg-120, and the catalytically labile hydrogen situated near Tyr-385.

The principal difference in the active sites of COX-1 and COX-2 is the presence in COX-2 of a side pocket off the main cyclooxygenase active site near the carboxylate binding region (34, 35). A comparable pocket is not accessible in COX-1 because of the substitution of Ile-523 for the Val-523 of COX-2 (36, 37). This side pocket is the binding site for the substituted sulfonamide or sulfone groups of the COX-2-selective inhibitors celecoxib and rofecoxib. Other differences between COX-1 and COX-2 in this region include the substitutions R513H and V434I. The side pocket is encoded by all COX-2 genes sequenced to date, yet no physiological consequence of this conserved active site difference has been elucidated. We constructed a triple mutant of COX-2, V523I/R513H/V434I, which comprises the major side pocket differences between the two enzymes, and we incubated it with 2-AG. The maximal rate of O₂ uptake by the mutant was $25 \pm 3\%$ ($n = 3$) of that observed with wild-type enzyme. When arachidonic acid was used as substrate, only minor differences were observed between the rates and extents of O₂ uptake catalyzed by the triple mutant relative to wild-type COX-2 incubated with arachidonic acid (data not shown). This suggests that one function of the side pocket is to enhance oxygenation of the ester substrate, 2-AG.

Cellular COX-2 Metabolism of Exogenous 2-AG—The ability of 2-AG to serve as a substrate for COX-2 *in vitro* raised the question of whether it could be oxidized in intact cells, where alternate metabolic enzymes, such as esterases, may compete with COX-2. The murine macrophage cell line, RAW264.7, was selected to address this question for several reasons. Unactivated RAW264.7 cells express no detectable COX-2 and low levels of COX-1, but IFN- γ and LPS induce COX-2 expression (38). The major arachidonic acid metabolite in these cells is PGD₂, so RAW264.7 cells permit the simultaneous evaluation



FIG. 5. Model of arachidonic acid bound in the COX-2 active site. Adapted from Ref. 23.

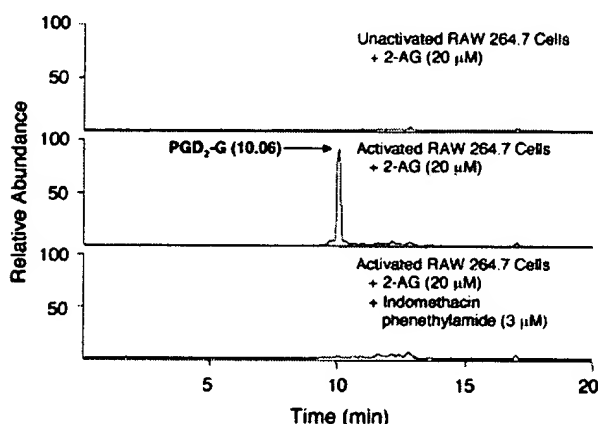


FIG. 6. 2-AG metabolism by activated RAW264.7 macrophages. PGD₂-G production and extracellular release by cells treated with exogenous 2-AG (20 μM) are shown. Products were eluted with a 15-min gradient of 20–100% acetonitrile in H₂O (0.001% sodium acetate). Chromatograms have been normalized to total ion current of 2-AG treated, activated macrophages.

of 2-AG oxygenation to PGH₂-G as well as endoperoxide metabolism by PGD synthase (26). Finally, the CB2 gene is transcribed in RAW264.7 macrophages, suggesting that cannabinoid signaling mechanisms are present and important in these cells (39).

Glycerol prostaglandins were not detectable in the medium from unactivated RAW264.7 cells after the addition of a physiologically relevant concentration of 2-AG (20 μM) (Fig. 6). However, cells treated with LPS (1 μg/ml) and IFN-γ (10 units/ml) synthesized and released PGD₂-G after the addition of 2-AG (Fig. 6). PGD₂-G biosynthesis was inhibited by both indomethacin (3 μM) and the highly selective COX-2 inhibitor, 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]-*N*-phenethylacetamide (indomethacin phenethylamide, 3 μM). No significant endogenous PGD₂-G biosynthesis was detectable in the absence of exogenous substrate under these conditions (Fig. 6 and data not shown). These studies demonstrate that 2-AG is a substrate for COX-2 in intact cells and that prostaglandin glycerol esters are released extracellularly. Furthermore, the absence of the nonenzymatic, PGH₂-G isomerization product, PGE₂-G, demonstrates that cellular PGD synthase metabolizes PGH₂-G readily.

Cellular COX-2 Metabolism of Endogenous 2-AG—The striking COX-2-dependent production of PGD₂-G by RAW264.7 cells treated with exogenous 2-AG prompted an extension of these studies into a more physiologically relevant setting. We investigated the possibility that cellular COX-2 oxygenates 2-AG released from endogenous cellular stores. 2-AG is generated *in vivo* through the Ca²⁺-dependent formation of *sn*-1-acyl-2-arachidonylglycerols followed by hydrolysis of the *sn*-1-acyl group by diacylglycerol lipase (14, 40). Ionomycin stimulation (5 μM) of IFN-γ/LPS-activated RAW264.7 cells resulted in the

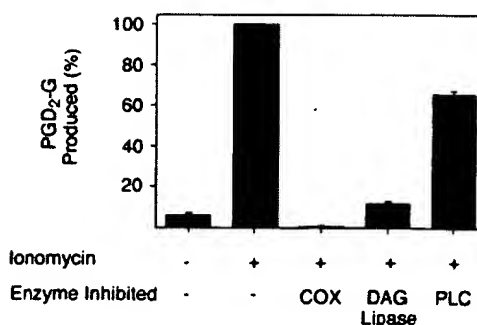
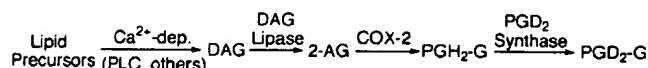


FIG. 7. Endogenous PGD₂-G production and extracellular release by ionomycin-stimulated, activated macrophages. Inhibitors were added 20 min prior to ionophore addition at the concentrations indicated under "Experimental Procedures." Values have been normalized to PGD₂-G production by uninhibited macrophages and represent the mean ± S.E. ($n = 6$, $p < 0.001$). Under tested conditions, uninhibited macrophages released 7.0 ± 0.1 ng of PGD₂-G/10⁶ cells (mean ± S.E., $n = 9$). Product identification was accomplished by coelution under multiple chromatographic conditions of macrophage product with both pentadeuterated PGD₂-G and the minor m/z 449 species produced by incubating 2-AG with purified COX-2.



SCHEME 2

production and extracellular release of PGD₂-G (Fig. 6). Quantification of PGD₂-G using a pentadeuterated standard revealed 7.0 ± 0.1 ng of PGD₂-G released/10⁶ cells (mean ± S.E., $n = 9$). Thus, activated RAW264.7 cells effect both the biosynthesis and COX-2-dependent oxygenation of the vasomodulatory endocannabinoid, 2-AG, upon calcium ionophore stimulation.

Endogenous PGD₂-G production was potentially inhibited by indomethacin (3 μM) and RHC-80267 (100 μM), a selective diacylglycerol lipase inhibitor (Fig. 7) (41). Both indomethacin and RHC-80267 effected dose-dependent inhibition of PGD₂-G biosynthesis (data not shown). In addition, the phospholipase C inhibitor U-73122 (5 μM) inhibited PGD₂-G biosynthesis by approximately 35% (Fig. 7) (42). Higher concentrations of U-73122 were not evaluated because of its low aqueous solubility. The moderate inhibition by U-73122 suggests a role for phospholipase C in DAG generation leading to PGD₂-G biosynthesis, but we cannot rule out a contribution from phospholipase D (43). Thus, PGD₂-G biosynthesis is calcium-dependent and results from the sequential actions of DAG lipase, COX-2, and PGD synthase (Scheme 2).

DISCUSSION

The present findings establish a function for COX-2 in the oxidation of the endogenous cannabinoid, 2-AG. The striking catalytic efficiency of COX-2 mediated 2-AG oxygenation coupled with evidence that 2-AG is the preferred ester substrate, even when compared with 1-AG, suggests that this endocannabinoid is a natural COX-2 substrate. Comparable oxygenation of 2-AG was observed with human and mouse COX-2, whereas 2-AG was a very poor substrate for sheep COX-1. This suggests that 2-AG oxygenation is a selective function of COX-2. Similar selectivity is observed in the COX-2-dependent oxygenation of anandamide (11). Indeed, in the present study, no glycerol prostaglandins were detected after incubation of 2-AG with unactivated RAW264.7 cells, which contain detectable levels of COX-1 (Fig. 6).

The identification of active site residues that promote efficient metabolism of 2-AG provides a molecular basis for isoform selectivity and suggests a *raison d'être* for the highly conserved

side pocket in COX-2 enzymes. Thus, in addition to differential regulation and tissue distribution, altered substrate specificity may represent an evolutionary impetus for the existence of two COX isoforms. Our initial studies only evaluated the role of residues that represent conserved differences between COX-2 and COX-1 in the side pocket region. There are other conserved differences between the two enzymes in the lobby region located below Arg-120, and their importance in the differential oxygenation of 2-AG is under evaluation.

The ability of intact cells to biosynthesize glyceryl prostaglandins when treated with exogenous 2-AG demonstrates that COX-2 may compete effectively for this endocannabinoid with other enzymes involved in its metabolism (e.g. esterases and acyltransferases). The detection of glyceryl prostaglandins in stimulated RAW264.7 cell medium, in the absence of exogenous 2-AG treatment, elucidates a 2-AG biosynthetic capacity in these macrophages and extends a recent observation that this endocannabinoid is produced in the murine macrophage cell line, J774 (40). More importantly, this observation demonstrates that endogenously generated 2-AG is a substrate for COX-2 action. Inhibition of PGD₂-G synthesis in macrophages by a DAG lipase inhibitor establishes a direct relationship between DAG production and glyceryl prostaglandin biosynthesis. This suggests that COX-2 occupies a central role in diverse signal transduction pathways involving DAG, endocannabinoids, and novel prostaglandin-like lipids.

The ability of the endoperoxide product of COX-2 action, PGH₂-G, to serve as a substrate for PGD synthase raises the possibility that 2-AG may be the precursor to a family of eicosanoids as diverse as those produced from arachidonic acid. Our finding that PGD₂-G is synthesized intracellularly and released extracellularly suggests that glyceryl prostaglandins are sufficiently stable to serve as intracellular or intercellular mediators. Evaluation of the binding of glyceryl prostaglandins to cell surface or nuclear receptors may provide insight into the mechanisms by which COX-2 produces manifold responses in both the central nervous and immune systems.

Finally, these findings establish a role for COX-2 in endogenous cannabinoid metabolism. Although anandamide oxygenation by COX-2 has been reported, previous investigations produced varied results, employed anandamide concentrations significantly higher than those present *in vivo*, and did not demonstrate oxygenation of anandamide released from endogenous cellular stores (11, 27, 44). The present findings establish that endogenously released 2-AG is oxygenated by COX-2 in agonist-stimulated macrophages. This suggests that COX-2 may play a regulatory role in endocannabinoid signaling by decreasing 2-AG levels and reducing tone at CB receptors (45, 46).

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